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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This research aims to examine differences of cancer-derived osteonectin from vascular-derived and bone cell-derived osteonectin. Of key interest is that these differences may contribute to the affinity of breast cancer cells to bone. During this first year of research, some of the structural differences between the two types of osteonectin have been identified. Breast cancer cells secrete a doublet of osteonectin that is larger than the vascular or bone cell-secreted osteonectin. In addition, breast cancer-secreted osteonectin does not appear to have N-linked glycosyl groups whereas the vascular and bone cell-derived osteonectin is N-linked glycosylated. We have designed three osteonectin affinity columns and have extracted 20-100 μg osteonectin from each cell type. All of these finding support the hypothesis that there are structural differences between breast cancer-secreted and vascular/bone-secreted osteonectin.

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bone-derived chemotactic factors, vasculature of bone, cell migration, cell culture model system, cell-cell interactions

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Introduction

Osteonectin is a secreted matrix protein that has a variety of functions, including cell adhesion modulation and chemoattraction. It has been implicated as a chemoattractant for breast cancer cells despite the evidence that some cancer cells secrete osteonectin. Other studies demonstrate the ability of osteonectin to permeabilize the vascular endothelial cell layer even though some endothelial cells also secrete osteonectin. This research aims to examine differences of cancer-derived osteonectin from vascular-derived and bone-derived osteonectin. Of key interest is that these differences may contribute to the affinity of breast cancer cells to bone. During this first year of research, some of the structural differences between the two types of osteonectin have been characterized.

Body

Task 1A, which was to determine the size of osteonectin derived from different cell type conditioned media, was accomplished by testing multiple cell lines for the production of osteonectin by both western blotting and ELISA. Conditioned media (about 250 mls) was collected from the osteonectin-secreting cell line. As shown in Figure 1, a human osteoblast line (hFOB1.19) and a human bone marrow endothelial line (HBME-1) both produce osteonectin of about 39kDa and 38kDa in size. The breast cancer cell lines, MDA-MB-435 and the MDA-MB-468 (not shown), secrete a larger form of osteonectin which migrates as a doublet with an upper band of about 41kDa and a lower band of about 38-39kDa respectively on SDS-PAGE. The breast cancer lines MDA-MB-231 and T47D do not secrete osteonectin. These observations were consistently found on all (10-30) western blots analyzed.

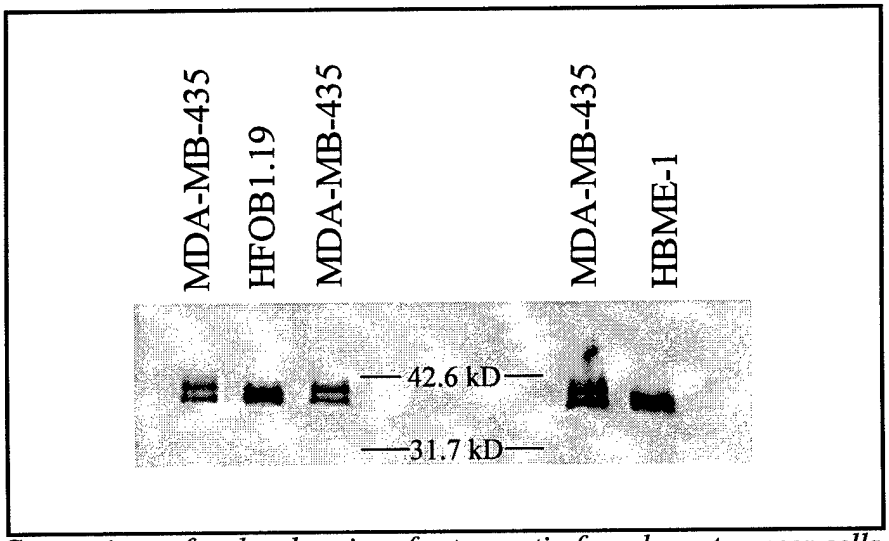

Figure 1 Comparison of molecular size of osteonectin from breast cancer cells and osteoblasts by immunoblotting. Conditioned serum–replacement medium (Sigma) was collected after 48 hours and separated by SDS-PAGE, transferred to nitrocellulose and then immunoblotted. Osteonectin secreted by MDA-MB-435 cells occurs as a doublet at ~ 41 kDa and 38 kDa. Osteonectin secreted by hFOB1.19 and HBME-1 cells is detected in a smaller doublet at ~ 39 kDa and 38 kDa.

Task 1B involved the purification of osteonectin from the previously mentioned cell lines. This was accomplished using the Pierce Aminolink® Plus affinity immobilization system instead of the column chromatography originally proposed. We obtained 20-100μg of pure osteonectin from each cell type. This required creating and optimizing a 50-fold scale-up method to remove osteonectin from 250ml verses a 5ml volume designed by the manufacturer.

To study the glycosylation of osteonectin proposed in Task 1C, a preliminary tunicamycin assay was performed. Tunicamycin stops cells from adding N-linked glycosyl groups to asparagine in the nascent protein chain. Cancer cells and bone cells were cultured in duplicate in the presence of tunicamycin at concentrations of 0, 25, 50, 100 and 200 ng/ml. It was found that the bone-secreted osteonectin contains N-linked glycosyl groups. Glycosylation of breast cancer cell osteonectin was not affected by tunicamycin and therefore is not glycosylated through the N-linkage. An enzymatic deglycosylation assay is being conducted to verify this finding as well as other types of glycosylation. Due to the length of time required to obtain the purified osteonectin (Task 1B), Task 1C was delayed, but is in progress.

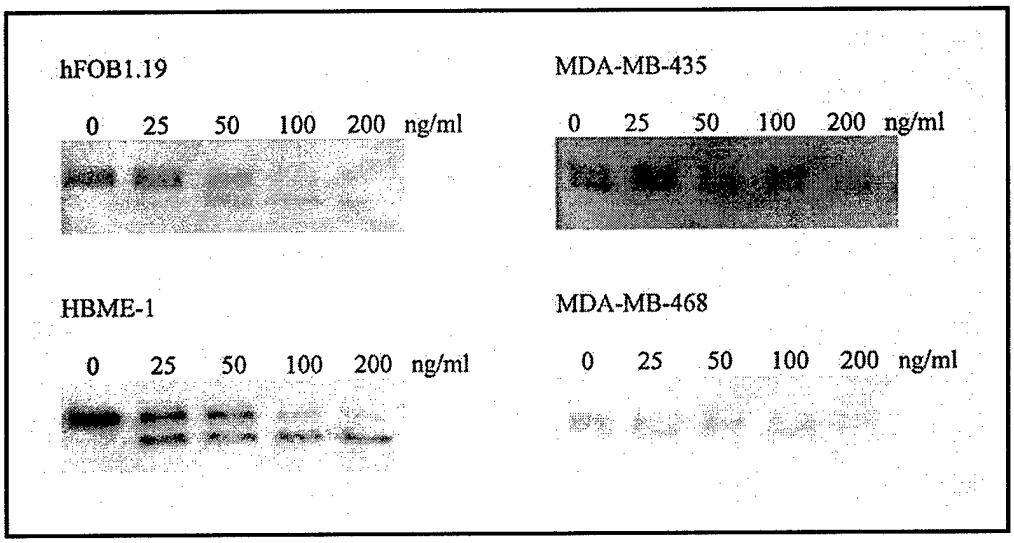

Figure 2 Comparison of secreted osteonectin from breast cancer and vascular/bone cell cultures treated with tunicamycin. Conditioned serum–replacement medium (Sigma) was collected after 24 hours from cultures treated with various tunicamycin concentrations (0,25,50, 100,200 ng/ml) and separated by SDS-PAGE, transferred to nitrocellulose and then immunoblotted. Alteration of bone cell (hFOB1.19) osteonectin is evident at the 50ng/ml dose and at the 25ng/ml dose for the vascular cells (HBME-1). All of the treated breast cancer cell-secreted osteonectin migrates the same as untreated controls.

In preliminary research for Task 3A, we have been unable to replicate assays showing that osteonectin influenced the chemoattraction of breast cancer cells. The migration assays consisted of placing breast cancer cells in the upper well of a Transwell chamber system with the different conditioned media samples placed in the lower chamber. Cells that migrated toward the conditioned media samples were counted. In seven replicates of

the migration assay, no significant chemoattraction toward osteonectin was found. It is possible that osteonectin may instead act to modify cell attachment (Murphy-Ullrich JE, 2001) thus rendering a cell more motile and therefore responsive to other chemoattractants.

Key Research Accomplishments

1. Collected conditioned media from the cancer cell lines (MDA-MB-435, MDA-MB-468, T47D and MDA-MB-231) and the vascular/bone cell lines (HBME-1 /hFOB1.19). Osteonectin was measured quantitatively by ELISA. (Task 1A)
2. Analyzed osteonectin samples on SDS-PAGE. Vascular and bone osteonectin doublet was about 39kDa and 38kDa in size. The cancer cell (MDA-MB-435, MDA-MB-468) osteonectin migrates as a larger doublet with an upper band of about 41kDa and a lower band of about 38-39kDa respectively. (Task 1A)
3. Designed three large scale osteonectin affinity columns and isolated and purified between 20-100 μg of osteonectin from each cell type. These osteonectin samples will be used in future analyses of osteonectin and its effects on cell migration and motility. (Task 1B)
4. Performed tunicamycin assay of osteonectin secreting cell lines to examine N-linked glycosylation. The cancer cells appear to lack N-linked glycosylation while the bone cells exhibit N-linked glycosylation. Other types of glycosylation are currently under investigation. (Task 1C)

Reportable Outcomes

1. Experience / training supported by this award:

   My academic training this year has included completing two courses in statistical analysis and use of statistical software. I have also participated in a weekly journal club in which current literature on breast cancer, apoptosis, and cell adhesion was presented and discussed. Each semester I attend, typically on a weekly basis, on-campus seminars dealing with a variety of disciplines such as neurobiology, cancer, stem cells, genetics, nutrition, and cell biology. For the past year, I have served as chairperson of the Penn State Physiology Student Forum; in this position I am responsible for inviting on-campus and off-campus scientists to present and discuss current topics in physiology with the graduate student group. In April 2002, I attended "Third North America Symposium on Skeletal Complications of Malignancy" in Washington D.C and delivered a poster presentation on my research (Campo, DA et al., 2002) this gave me the opportunity to meet and interact with other researchers in the field of cancer metastasis. In the laboratory, I have mastered many new techniques including ELISA, Affinity Column preparation and protein isolation, confocal and interference reflection microscopy.

2. Poster presentation

   Campo DA, DM Sosnoski, AM Mastro, DR Welch, CV Gay. Differences between osteoblast-secreted and breast cancer-secreted osteonectin: N-linked glycosylation may be key in chemoattraction. Oncology, 17(Suppl 3): p 20, 2003. This was presented at "Third North America Symposium on Skeletal Complications of Malignancy"

Conclusion

The research completed in the last year has supported the hypothesis that there are structural differences between breast cancer-secreted and vascular/ bone-secreted osteonectin. This has been demonstrated by size analysis on western blots and by N-linked glycosylation assays. Further studies need to be completed to better identify and detail the chemical differences between the two types of secreted osteonectin. Research will also be undertaken to investigate the effects of osteonectin in differentially glycosylated forms on cell motility.

References

Campo DA, DM Sosnoski, AM Mastro, DR Welch, CV Gay. Differences between osteoblast-secreted and breast cancer-secreted osteonectin: N-linked glycosylation may be key in chemoattraction. Oncology, 17(Suppl 3): p 20, 2003.

Murphy-Ullrich JE. The de-adhesion activity of matricellular proteins: Is intermediate cell adhesion an adaptive state? J Clin Invest. 7: p 785-790, 2001.

Appendix

Differences between osteoblast-secreted and breast cancer-secreted osteonectin: N-linked glycosylation may be key in chemoattraction.

[1]D.A. Campo, [2]D.M. Sosnoski, [2]A.M. Mastro, [3]D.R. Welch, [1,2]C.V. Gay.
[1]Intercollege Program in Physiology and [2]Department of Biochemistry and Molecular Biology, Penn State University, University Park, PA and [3]Jake Gittlen Cancer Research Institute, Penn State University School of Medicine, Hershey, PA.

Osteonectin, first identified in bone, has wide tissue distribution, varying degrees of glycosylation and has been shown to be a chemoattractant for breast cancer cells. Here we report differences between bone-derived osteonectin and breast cancer-derived osteonectin and show differential chemoattraction. In one experiment, individual cultures of the human fetal osteoblast cell line (hFOB1.19) and a metastatic breast cancer cell line (MDA-MB-435) were grown with or without tunicamycin, a potent inhibitor of N-linked glycosylation. Conditioned, serum-free media (CM) were collected from the cultures. Aliquots of CM were subjected to SDS-page gel electrophoresis and blotted on a nitrocellulose membrane. Immunostaining with mouse anti-human osteonectin was used to detect osteonectin bands. In untreated hFOB1.19 cells, a doublet of osteonectin (~39kDa and ~38kDa) was detected; the MDA-MB-435 cells also secreted osteonectin of two sizes (~41kDa and ~38kDa). Upon treatment with tunicamycin, the hFOB1.19 doublet decreased in size (~36kDa and ~35kDa), whereas the MDA-MB-435 osteonectin was unchanged. The data show that osteoblast-derived osteonectin is heavily glycosylated through the N-linkage, whereas osteonectin from breast cancer cells has no detectable N-linked glycosylation. One consequence of altered glycosylation is a change in protein folding which could account for different chemotactic potentials of osteonectin. In another experiment which was designed to assess the chemotactic potential of the two forms of osteonectin, breast cancer cells ($5X10^5$ cells/well) were placed in the upper chamber of a transwell chamber insert (12μm pore size) coated with Matrigel®. CM from untreated hFOB1.19 or MDA-MB-435 cells was placed in the lower chamber. After 48 hours, the number of MDA-MB-435 cells migrating through the transwell membrane toward the hFOB1.19 CM was 4-fold greater than toward MDA-MB-435 CM. Collectively, the results indicate that bone-derived osteonectin is distinct from the breast cancer osteonectin in molecular weight and glycosylation. Furthermore, bone-derived osteonectin has an enhanced chemotactic potential for breast cancer cells.

Published in Oncology, 17(Suppl 3): p 20, 2003.